

Immunophenotyping without antibodies

New perspectives for lymphoma characterization

The current WHO classification of haematopoietic neoplasms integrates genetic and chromosomal aberrations as well as consecutive protein changes [2]. Haematopoietic neoplasms are mainly defined morphologically according to their normal counterpart in reactive lymphoid and bone marrow tissue. However, many overlapping histological features exist in cancerous tissue, making antibody-based techniques indispensable to classify lymphomas and to increase diagnostic accuracy. Immunohistochemistry (IHC) is the most important method in routine diagnostics today. Although antibody-based methods allow lymphomas to be classi-

fied to a certain extent, there are still many “grey zones” with overlapping morphological and immunophenotypic features, making it difficult to determine lymphomas exactly [6, 7]. Moreover, among the currently available markers used, prognostic and predictive protein markers are still scarce, and the present classification seems inadequate in predicting the outcome of each lymphoma type [3, 4, 5].

The molecular composition and dynamic organization of the plasma membrane (PM) determines how a cell can interact with its environment at any given moment [9]. The identification of predictive and diagnostic PM markers for cancer is

particularly challenging, because neoplastic cells largely share the same genome and proteome as normal cells. Microarray-based mRNA expression profiling experiments can roughly estimate the expression of a protein by measuring the protein encoding mRNA, but they cannot predict protein localization or post-translational modifications. In order to understand and unravel cancer biology at the molecular level, the PM protein composition as well as post- and co-translational modifications must be investigated directly. Since PM proteins are accessible for diagnostic and therapeutic intervention by antibodies or small molecule-based strategies, these protein targets are of the greatest interest as potential diagnostic and predictive markers.

Challenges to identifying the PM subproteome arise from the need to overcome the technical difficulties in isolating PM proteins, the limited relative abundance of PM proteins and the difficulty in resolving and identifying hydrophobic polypeptides. To address these issues, we have developed new methodologies for the specific labelling of proteins residing in the PM to circumvent the above-mentioned problems [8]. Our approach exploits the fact that most cell surface proteins are glycosylated. Glycosylation of PM proteins is a common co-translational modification [10]. The highly specific CSC technology utilizes a three-step tandem affinity la-

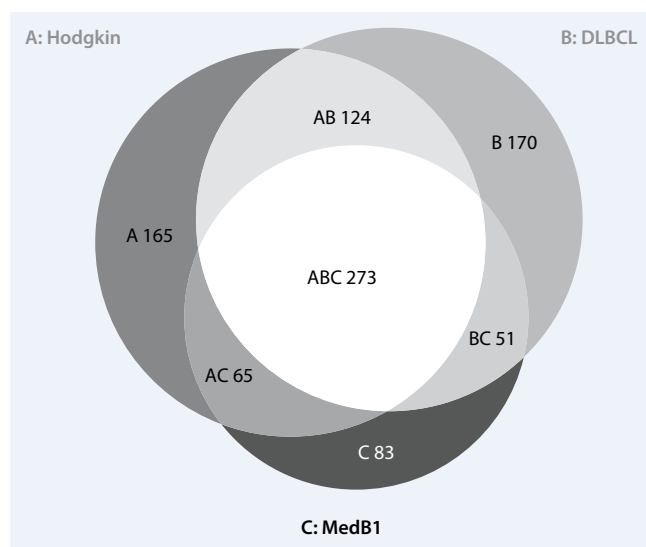


Fig. 1 ◀ Overlapping and differentially expressed TM proteins identified by CSC technology

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**Immunphänotypisierung ohne Antikörper.
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Zusammenfassung

Ziel. Eine exakte Klassifikation hämatologischer Neoplasien ist Voraussetzung für eine korrekte Diagnose, Prognose und Therapiestrategie. Die Lymphomklassifikation war bis dahin limitiert durch die beschränkte Verfügbarkeit von Antikörpern gegen Oberflächenproteine, die in der Routinediagnostik an Paraffinmaterial anwendbar sind. Eine systematische und quantitative Analyse von Oberflächenproteinen ist notwendig, um neue Proteinmarker auf Lymphomsubtypen zu identifizieren.

Methode. Neun Hodgkin- und Non-Hodgkin-B-Zelllinien vom diffus großzelligen und mediastinalen Typ wurden mittels sog. „Cell surface capture“ (CSC)-Technologie, einer massenspektrometrie-basierten Methode, nach Zelloberflächenglykoproteinen untersucht. Selektionierte Proteine wurden mittels antikörperbasierter Methoden unter Einschluss von Durchflusszytometrie und Immunhistochemie auf Gewebeschips validiert.

Resultate. Insgesamt wurden 747 Transmembranproteine identifiziert, davon 142 CD- („cluster of differentiation“) anno-

tierte Proteine. Mittels quantitativer CSC-Technologie wurde eine Gruppe unterschiedlich exprimierter Oberflächenglykoproteine zwischen Hodgkin- und Non-Hodgkin-B-Zelllinien nachgewiesen. Dabei wurden sowohl typische CD-Moleküle wie CD20 und CD30, aber auch weniger häufige Moleküle wie CD2 in Hodgkin-Lymphom- (HL-)Zelllinien identifiziert und mittels Immunhistochemie auf primären Zelllinien und Lymphomgewebe validiert. Ein Set von differenziell exprimierten Glykoproteinkandidaten wird aktuell auf Lymphomgewebeschips untersucht.

Schlussfolgerung. Die CSC-Technologie erlaubt, gleichzeitig mehrere hundert Oberflächenproteine auf Lymphomzelllinien nachzuweisen. Darüber hinaus können auch unbekannte, differenziell exprimierte Proteine identifiziert werden.

Schlüsselwörter

Zelloberflächenglykoproteine · „Cell surface capture“ (CSC)-Technologie · Massenspektrometrie · Lymphom

Immunphenotyping without antibodies. New perspectives for lymphoma characterization

Abstract

Aims. Accurate classification of haematological malignancies is a prerequisite for their correct diagnosis, prognosis and therapy. Clear classification of lymphomas is often hindered by the limited number of available cell surface protein markers that are suitable for immunophenotyping. A systematic and quantitative analysis of cell surface proteins is thus required to identify new protein markers on lymphoma subtypes in an unbiased and discovery-driven approach.

Methods. Nine Hodgkin and non-Hodgkin B cell lines of diffuse large cell type and mediastinal type were investigated by cell surface capture (CSC) technology, a mass spectrometry-based method to identify cell surface glycoproteins. Selected proteins are verified by antibody-based methods, including flow cytometry and immunohistochemistry on cell line arrays.

Results. A total of 747 predicted transmembrane proteins were identified from all cell

lines, including 142 CD (cluster of differentiation) annotated proteins. A group of differentially expressed cell surface glycoproteins between Hodgkin and non-Hodgkin B cell lines was revealed via quantitative CSC technology. In addition to classical and expected CD molecules such as CD20 and CD30, less frequently expressed molecules such as CD2 on Hodgkin lymphoma (HL) cell lines were identified by CSC and verified by immunohistochemistry in cell lines and primary lymphoma tissue. A panel of CSC-identified differentiation glycoprotein candidates is currently under investigation on tissue microarrays (TMAs) from patient samples.

Keywords

Cell surface glycoprotein markers · Cell surface capture (CSC) technology · Mass spectrometry · Lymphoma

bellung strategy to confer the desired specificity for glycoproteins in the PM subproteomes. Results from model cell lines and from primary cells and tissues have yielded a degree of specificity never seen before in the detection of low-abundance PM proteins, with less than 10% contamination from intracellular and non-glycosylated peptides/proteins. The “contaminations” can be singled out in a bioinformatics approach, yielding 100% *bona fide* PM proteins. CSC technology uses the mass spectrometer as an “antibody” capable of specifically detecting PM glycoproteins that cannot currently be detected any other way.

Materials and Methods

Four HL cell lines of nodular sclerosing and mixed cellularity type, four diffuse large B cell lymphoma (DLBCL) lines of germinal centre and activated cell type and a mediastinal B cell lymphoma (MedB1) line were investigated via mass spectrometry by CSC technology. The cell surface proteomes of the cell lines were compared, and selected differentially expressed proteins were validated on the single cell level with affinity-based probes on a formalin-fixed and paraffin-embedded cell line array of the same set of cell lines. Validated protein targets are further analysed on newly established lymphoma TMAs from patient samples.

Results

A total of 747 predicted transmembrane proteins were identified in all cell lines, including 142 CD molecules (■ Tab. 1). Many of the identified proteins are well-known CD molecules such as CD19, CD20 and CD22 in the DLBCL lines or CD30 in the HL and MedB1 lines. CSC technology also revealed CD molecules that are low abundant in lymphoma tissue such as CD2 in the HL lines HDLM2 and L540. Whereas some of the transmembrane proteins are differentially expressed, others overlap between different lymphoma subtypes (■ Fig. 1). Based on all identified predicted transmembrane proteins, unsupervised hierarchical clustering using SPOTFIRE software showed a cluster of DLBCL lines that was separated from the

Tab. 1 Number of TM-predicted and CD-annotated proteins identified in Hodgkin's lymphoma, mediastinal large B cell lymphoma and diffuse large B cell lymphoma

Cell lines	Number of unique pre-dicted TM and GPI-anchored proteins	Number of unique CD proteins
HL_HDLM-2	332	85
HL_KMH-2	337	78
HL_L-428	445	80
HL_L-540	348	87
K_MedB1	497	98
NH_HBL-1	355	88
NH_SU-4	359	80
NH_SUDHL-6	347	89
NH_U2932	352	100
All records	986	177

group of HL lines. In contrast, the MedB1 line was clearly split up with these two groups. Though the MedB1 line seems to be a distinct entity, it is more closely related to the HL than to the DLBCL lines. Together, the CSC data disclose the cell surface proteomes of HL, DLBCL and MedB1 lines in great detail and allow for novel insights into the functional capacities of these cells.

Discussion

CSC technology is a mass spectrometry-based technology that allows for the discovery-driven identification of cell surface proteins regardless of the availability of antibodies. The proteomic data generated correlate with findings from gene array analysis regarding the relatedness of Hodgkin's and mediastinal large B cell lymphoma (MedB1) cell lines [3, 1, 6]. Moreover, the CSC results revealed overlapping and differentially expressed known and unknown transmembrane proteins in the different cell lines. By validating differentially expressed proteins on the investigated cell lines and on primary lymphoma tissue, we aim to identify new specific markers to facilitate the differential diagnosis of lymphomas.

As we were recently also able to apply the CSC technology to primary tissue, subproteome-targeted proteomics opens

up new perspectives for a refined classification of lymphomas and future potential targets for specific treatment approaches.

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Conflict of interest. The corresponding author certifies that no actual or potential conflict of interest exists in relation to this article.

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